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A Comprehensive Approach for Quantitative Lignin Characterization by NMR Spectroscopy

EWELLYN A. CAPANEMA,* MIKHAIL Y. BALAKSHIN, AND JOHN F. KADLA§

Department of Wood and Paper Science, North Carolina State University, Raleigh, North Carolina, 27695-8005

A detailed approach for the quantification of different lignin structures in milled wood lignin (MWL) has been suggested using a combination of NMR techniques. $^{1}H-^{13}C$ heteronuclear multiple quantum coherence and quantitative ^{13}C NMR of nonacetylated and acetylated spruce MWL have been found to have a synergetic effect, resulting in significant progress in the characterization of lignin moieties by NMR. About 80% of side chain moieties, such as different β-O-4, dibenzodioxocin, phenylcoumaran, pinoresinol, and others, have been identified on the structural level. The presence of appreciable amounts of α-O-alkyl and γ-O-alkyl ethers has been suggested. Although the quantification of various condensed moieties was less precise than for side chain structures, reliable information can be obtained. Comparison of the calculated results with known databases on spruce MWL structure shows that the suggested approach is rather informative and comparable with the information obtained from the combination of various wet chemistry methods. Discrepancies between the results obtained in this study and those previously published are discussed.

KEYWORDS: Lignin; milled wood lignin (MWL); quantitative ^{13}C NMR spectroscopy; HMQC NMR technique

INTRODUCTION

Lignin is one of the major polymers occurring in the plant kingdom. Despite extensive investigation, the complex and irregular structure of lignin is not completely understood. Analysis of lignin with different wet chemistry techniques and model studies on dehydrogenative polymerization of coniferyl alcohol has resulted in different models of lignin structure (*1*−*5*) (**Table 1**). Wet chemistry methods such as functional group analysis and degradation techniques can be very precise for specific functional groups and structural moieties. However, each technique gives limited information and is not able to provide a general picture of the entire lignin structure. Moreover, degradative techniques are indirect methods. Conclusions regarding the original lignin structure are deduced from the analysis of degradation products, in which the origin is sometimes ambiguous, and correction coefficients are often needed for quantitative calculations (*5*).

The advantage of spectroscopic methods over degradation techniques is the analysis of the whole lignin structure and direct detection of lignin moieties. The advantage of nuclear magnetic resonance (NMR) spectroscopy over other spectroscopic techniques, such as infrared (IR), ultraviolet−visible (UV), and Raman spectroscopy, is that NMR has a much higher resolution, enabling a larger amount of information to be obtained. The development of quantitative ^{13}C NMR in lignin analysis (*6*) was an important milestone in lignin chemistry. However, despite the wide use of this technique for the characterization of lignin structure, there are few comprehensive works on lignin quantification with ^{13}C NMR (*8*−*12*). Quite often, ^{13}C NMR is used only for the estimation of some specific moieties (*13*−*16*). Very recently, a quantitative two-dimensional (2D) NMR approach has been suggested (*15*, *17*, *18*). However, this method requires some experimental precautions, which presently make it rather difficult to perform. In addition, the HMQC technique does not allow detection of quaternary carbon. Quantification with the DEPT technique (*7*, *19*) is not a simple approach either and requires very tedious work on experimental conditions to be quantitative. Therefore, quantitative ^{13}C NMR is still the most used NMR method for lignin characterization, being rather informative, reliable, and at the same time relatively feasible.

Detailed methods on the characterization of spruce MWL with ^{13}C NMR were suggested in the late 1980s. Since that time, appreciable progress has been made in NMR spectroscopy in general and in lignin characterization in particular; the quality of spectrometers and software appreciably improved allowing significant improvement in spectra quality and quantification. The database of lignin model compounds has been further expanded (*20*). In addition, recent development in multidimensional NMR techniques (*21*) has provided new and important information on lignin structure. Using these recent advances in NMR analysis of lignin structure, further development in the quantitative analysis of lignin can be made.

The general goal of our work was to develop an independent and reliable approach for the quantification of lignin moieties by NMR spectroscopy. This was accomplished by the critical

* To whom correspondence should be addressed. Tel: 919-515-5797. Fax: 919-515-6302. E-mail: capanema@unity.ncsu.edu.
§ Current address: Department of Wood Science, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4.

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Table 1. Comparison of the Results Obtained with Different Structural Models for Spruce MWL and Different NMR Approaches

	comprehensive models (moieties/100 C_9 units)			NMR calculations (moieties/100 Ar)			
units	ref *1*	ref *2*	ref *3*	ref *9*	ref *10*	ref *17*	present
			structures				
β-O-4/α-OH			34			36	36
β-O-4/α-CO				3		<1	2
DBDO				-		5	7
phenylcoumaran	9	9–12	10	9		12	9
pinoresinol			3.5			2.5	2
β-1/α-OH		7	9			2	1
spirodienone						2	2
secoisolariciresinol						1	1
coniferyl alcohol			4			1	2
dihydroconiferyl alcohol			4			1	2
Ar-CO-CH_2-CH_2OH						3	2
vanillin				4		3	5
coniferaldehyde		4	4			5	4
			interunit linkages				
β-O-4 total	44	50	45	53	53		45[b]
α-O-4 total	17				14		16[b]
with β-O-4 (noncyclic)	8	6–8	11				<1
alkyl-O-alkyl total[a]	18						39–45
α-O-alkyl total[a]							15–21
α-O-γ			7				
γ-O-alkyl[a]							24
β-β total[a]		4	14				6[b]
β-5 total			14				
β-6 (β-2)		3	4				
5-5′ total[a]		19–22	21	24–26		22	24–27
etherified[a]				19–21			19
phenolic[a]				4–6			5–8
4-O-5	6	4	7		9		
			functional groups				
total OH	132		131				138
aliphatic OH	100		100				107
primary	75				78		68
secondary	25						39
benzylic		16	25				38
p-alkoxy-		10	18				
p-hydroxy-		6	7				
phenolic OH	32	20–25	31		34		31
total carbonyl	20	20	21		46	15	21
aldehyde	3				9		9
ketone	17				37		10
α-CO		6–8	6				10
nonconjugated		10	11				5
COOR total	4		2		28		5
conjugated							2
aliphatic							3
COOH					5		
esters	4		2		23		
degree of condensation			45	44	56[c]		38

[a] Amount of C_9 units involved; in a case of symmetric moieties, such as α-O-α, γ-O-γ, and β-β, the amount of these structures will be half of the value present. [b] Sum of identified moieties. [c] Calculated based on data (*10*) as sum of condensed C-C and Ar-O-Ar. The authors considered only C-C moieties (0.48/Ar) as condensed.

evaluation of published results on lignin quantification and by further development of the calculation method. To verify the calculation algorithm and correlate the data obtained by the advanced calculation method with information available on lignin structure, a well-investigated lignin, spruce MWL, was used.

MATERIALS AND METHODS

Preparation of MWL. Spruce (*Picea abies*) sawdust (fraction < 60 mesh) was extracted with ethanol/toluene, 1:2 (v/v). The extracted sawdust was then subjected to dry milling (48 h) in a zirconium planetary ball mill under nitrogen atmosphere followed by dioxane extraction. The preparations obtained underwent purification common for MWL (*22*). The yield of the purified MWL was 25% per Klason lignin in wood. The amount of carbohydrates in the MWL was <0.7%.

Acetylation of the purified MWL was carried out according to a published procedure (*23*). No purification of the acetylated preparation was used.

NMR Analysis. The NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer at 300 K using DMSO-d_6 as the solvent. Chemical shifts were referenced to TMS (0.0 ppm). The HMQC analysis was performed with ca. 5% solution of lignin applying a 90° pulse width, a 0.12 s acquisition time, a 1.0 s pulse delay, and $^1J_{C-H}$ of 147 Hz. For the quantitative ^{13}C NMR, the concentration of lignin was 20%; a 90° pulse width, a 1.4 s acquisition time, and a 1.7 s relaxation delay were used. Chromium(III) acetylacetonate (0.01 M) was added to the lignin solution to provide complete relaxation of all nuclei (*10*, *19*). A total of 20 000 scans were collected. A quantitative ^{1}H NMR spectrum of acetylated MWL was recorded at a lignin concentration of ~2% in $CDCl_3$, with a 90° pulse width and a 1.3 s acquisition time.

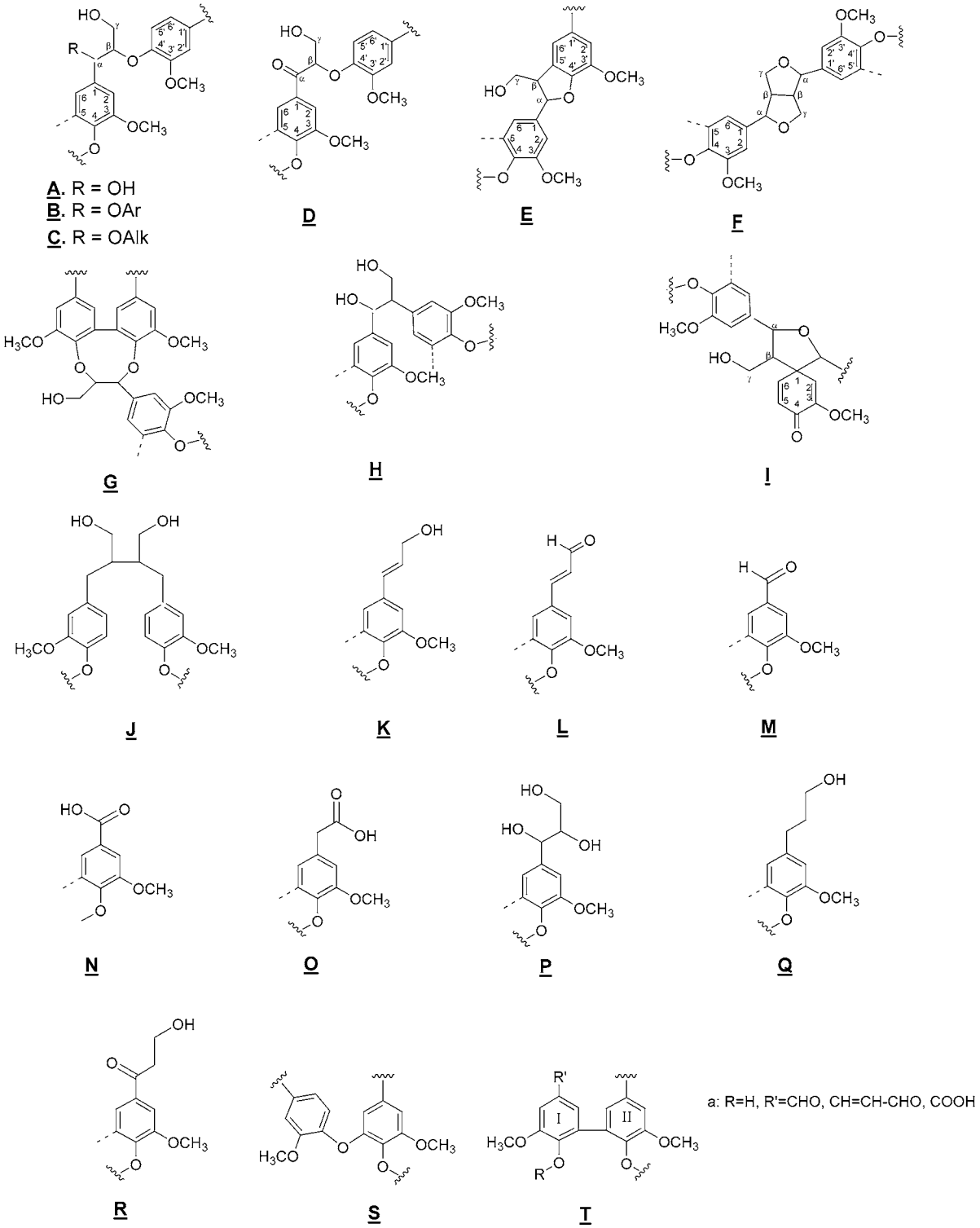

Figure 1. Lignin substructures.

A relatively high relaxation delay of 7 s was applied to ensure complete relaxation of aldehyde protons. A total of 128 scans were collected.

RESULTS AND DISCUSSION

Integrated Use of the HMQC and Quantitative ^{13}C NMR Techniques. The first attempt of integrated use of 2D and quantitative NMR was made recently (*24*). Unfortunately, the sensitivity of the HETCOR technique was very low for lignin analysis, and only very few lignin moieties have been detected. The sensitivity of the HMQC technique is much higher providing the information on the presence/absence of specific lignin moieties (**Figure 1**) in the lignin preparations and making analysis of the lignin structure with quantitative ^{13}C NMR easier and more accurate. Although signals of some lignin structures are overlapped in the ^{13}C NMR spectra (**Tables 2** and **3**), in most cases, it is possible to calculate the quantity of different units by solving sets of mathematical equations (**Table 4**).

Acetylated lignin preparations are often considered not fully representative of the original lignin, as some of the material is typically lost during isolation, resulting in lignin fractionation. However, the isolated yield of acetylated MWL using the method chosen (*23*) was close to theoretical (about 130 mass%).

Table 2. Signal Assignment in the NMR Spectrum of Nonacetylated MWL

no.	range (ppm)	assignment	amount (per Ar)
1	210–200	nonconjugated CO	0.05
2	200–196	α-CO except **D**	0.04
3	196–193	CO in α-CO/β-O-4 (**D**), **L**	0.06
4	193–191	Ar-CHO (**M**)	0.05
5	182–180	C-4 in **I**	0.02
6	175–168	aliphatic COOR	0.03
7	168–166	conjugated COOR	0.02
8	162–160	C-4 in h-units	0.02
9	157–151	C-3 in $\mathbf{T}_{et}$, C-3,5 in $\mathbf{S}_{et}$, C-α in **L**, C-3,6 in **I**, C-4 in conjugated CO/COOR etherified units	0.37
10	144.5–142.5	C-3 in **E**, C-4 in $\mathbf{T}_{ne}$, C-4 in conjugated **S**, unknown	0.18
11	58–54	OMe, C-β in **H**, C-1 in **I**, C-γ in **R**	1.00
12	54–52	C-β in **E** and **F**	0.13
13	35–34	C-β in **Q**, C-α in **J**	0.04
14	32.5–31.5	C-α in **Q**	0.02
		clusters	
	162–142	"C_{Ar-O}"	2.08
	142–125	"C_{Ar-C}"	1.55
	125–102	"C_{Ar-H}"	2.50
	90–58	Alk-O-	2.12
	90–77	Alk-O-Ar, α-O-Alk	0.81
	77–65	γ-O-Alk, OH_{sec}	0.63
	65–58	OH_{prim}	0.68

Table 3. Signal Assignment in the NMR Spectrum of Acetylated MWL

no.	range (ppm)	assignment	amount (per Ar)
1a	210–200	nonconjugated CO	0.03
2a	200–196	α-CO except **D**	0.02
3a	196–193	CO in α-CO/β-O-4 (**D**), **L**	0.06
4a	193–191	Ar-CHO (**M**)	0.05
5a	182–180	C-4 in **I**	0.015
6a	172–169.6	primary aliphatic OH	0.68
7a	169.6–168.6	secondary aliphatic OH	0.39
8a	168.6–166	phenolic OH, conjugated COOR	0.33
9a	162–160	C-4 in h-units etherified	0.01
10a	162–148	all C-3 (except **E** and h-units), C-5 in **S**, C-α in **L**, C-6 in **I**, C-4 in conjugated CO/COOR etherified and h-units	1.07
11a	144.5–142.5	C-3 in **E**, C-4 in conjugated $\mathbf{S}_{et}$, unknown	0.19
12a	88–86	C-α in **E**	0.09
13a	86–83	C-α in **F**, **G**	0.11
14a	83–81.5	C-β in **G**	0.10
15a	77–72.5	C-α in **A**, **H**, **P**, carbohydrates	0.40
16a	60–59	C-γ in **R**	0.02
17a	58–54	OMe, C-1 and C-β in **I**	0.99
18a	50–48	C-β in **H**, **E**	0.10
19a	35–34	C-α in **J**	0.02
20a	32.5–31.5	C-α in **Q**	0.02
		clusters	
	125.5–103	"C_{Ar-H}"	2.60
	90–58	Alk-O-	2.17
	90–77	Alk-O-Ar, α-O-Alk	0.83
	77–65	γ-O-Alk, OH_{sec}	0.64
	65–58	OH_{prim}	0.70

The quality of the ^{13}C NMR spectrum of the acetylated MWL preparation was rather good (**Figure 3**); therefore, to avoid the loss of material and the potential change in chemical composition (*23*), no purification procedure was used. To avoid any variation in the chemical shifts of lignin signals due to different solvents and to remove the solvent signal from important areas of the lignin spectra, for example, δ_C 77.2 for $CDCl_3$, DMSO was used for both the nonacetylated and the acetylated lignin preparations. Good correlation between the nonacetylated MWL and the MWL-Ac was confirmed by the very close values for the integrals in the three main clusters of signals at 90−77, 77−66, and 65−57.5 ppm and other areas (**Tables 2** and **3**).

Detailed information on the chemical shifts of different lignin moieties along with comprehensive peak assignments in the HMQC and ^{13}C NMR spectra of lignins and DHP can be found in different publications (*8*, *9*, *20*, *21*, *25*−*29*). Therefore, we list only signals used for quantification (**Tables 2** and **3**).

Optimization of Quantitative ^{13}C NMR Experiment. Usually, quantitative ^{13}C NMR requires an appreciable amount of lignin (200−600 mg) and a long experimental time (about 72 h) to obtain spectra of good quality (*8*, *9*). Significant experimental time results from relatively long pulse delays are needed to provide complete relaxation of all nuclei. The relaxation time can be significantly reduced by addition of a relaxant to the lignin solution. The use of $Cr(acac)_3$ in quantitative ^{13}C NMR of lignins has been reported earlier (*11*, *12*, *19*), but the concentration of the relaxant was different. However, it has not been shown experimentally that addition of the relaxant at the concentration used does not affect the quality of the spectra. Our results have demonstrated that the spectra of the spruce MWL obtained under the classical conditions without relaxant and with 0.01 M relaxant with much shorter pulse delays were practically the same (**Figure 3**). No line broadening was observed, and the integral values were practically the same. Therefore, the use of 0.01 M $Cr(acac)_3$ does not affect the quality of the ^{13}C NMR spectra and allows a 4-fold decrease in the experimental time. Another improvement was achieved by the use of a Shigemi microtube, which allows a 2−3-fold decrease in the amount of sample by more efficient tube configuration. Thus, quantitative^{13}C NMR spectra can be obtained within 18 h with 50−70 mg of lignin.

Accuracy of NMR Measurements and Calculations. The accuracy of integration of strong and well-resolved peaks and cluster of signals, such as total OH and OMe groups and total aromatic and total oxygenated aliphatic carbons, was estimated as ∼±2% at only 2000 scans (*30*) and should be even better with 20 000 scans collected. The accuracy of the integration of partially resolved peaks as well as calculation using subtraction of experimental data, which are significantly higher than the resulting calculated value, will be lower but still useful. It is important to avoid, if possible, "chain calculation" when an error obtained in the original step results in errors in consequent calculations. The use of several independent methods for the quantification of different moieties significantly increases the reliability of their estimation.

Estimation of Different Lignin Moieties from Quantitative ^{13}C NMR Spectra. The integral of the 162−102 ppm region was set as the reference, assuming that it includes six aromatic carbons and 0.12 vinylic carbons (*9*). It follows that the integral value divided by 6.12 is equivalent to one aromatic ring (Ar).

Carbohydrate Impurities. Sugar analysis showed that the amount of carbohydrates in the MWL is negligible, <0.7%. This is consistent with very weak sugar signals in the HMQC spectra (**Figure 2**) and a very small resonance of C-1 of carbohydrates at 102−90 ppm (**Figure 3**). Therefore, carbohydrates do not interfere with the analysis of lignin moieties by ^{13}C NMR in our MWL preparation.

Quantification of Side Chain Moieties on the Structural Level. Estimation of lignin moieties on the structural level is very important allowing more comprehensive information about lignin architecture and reactivity. NMR spectroscopy, especially correlation techniques, enables the majority of lignin side chain

Table 4. Estimation of Various Lignin Moieties by ^{13}C NMR

structure	calculation	value (per Ar)
vanillin (**M**)	$(I_{193-191})_{na}$	0.05
	$(I_{193-191})_{ac}$	0.05
spirodienone (**I**)	$(I_{182-180})_{na}$	0.02
	$(I_{182-180})_{ac}$	0.015
phenylcoumaran (**E**)	$(I_{88-86})_{ac}$	0.09
pinoresinol (**F**)	$(I_{54-52})_{na} - \mathbf{E}$	0.04[b]
β-1 (**H**)	$(I_{50-48})_{ac} - \mathbf{E}$	0.01
dibenzodioxocin (**G**)	$(I_{86-83})_{ac} - \mathbf{F}$	0.07
α-CO/β-O-4 (**D**)	$(I_{196-193})_{na} - \mathbf{L}$[a]	0.02
	$(I_{196-193})_{ac} - \mathbf{L}$[a]	0.02
β-O-4/α-OH (**A**)	$(I_{77-72.5})_{ac} - \mathbf{H} - \mathbf{P}$[a] $- 3 \times$ (sugar)	0.36
dihydroconiferyl alcohol (**Q**)	$(I_{32.5-31.5})_{na}$	0.02
	$(I_{32.5-31.5})_{ac}$	0.02
secoisolariciresinol (**J**)	$(I_{35-34})_{ac}$	0.02[b]
	$(I_{35-34})_{na} - \mathbf{Q}$	0.02[b]
Ar-CO-CH_2-CH_2OH (**R**)	$(I_{60-59})_{ac}$	0.02
p-hydroxyphenyl (h-units)	$(I_{162-160})_{na}$	0.02
OMe	$(I_{58-54})_{na} - \mathbf{H} - \mathbf{I} - \mathbf{R}$	0.95
	$(I_{58-54})_{ac} - 2 \times \mathbf{I}$	0.95
OH_{ph}	$(I_{168.6-166})_{ac} - (I_{168-166})_{na}$	0.31
total etherified	$1.00 - OH_{ph} - \mathbf{I}$	0.67
Alk-O-Ar (ident.)	$\mathbf{A} + \mathbf{D} + \mathbf{E} + 2 \times \mathbf{G}$	0.61
γ-O-Alk	$I_{77-65} - OH_{sec}$	0.24
α-O-Alk maximum	I_{90-77} – Alk-O-Ar	0.21
minimum	$I_{90-77} - (1.00 - OH_{ph} - \mathbf{I})$	0.15
side chain	$I_{90-45} - OMe + 2 \times (\mathbf{J} + \mathbf{Q}) + \mathbf{R} + \mathbf{O} + 2 \times (\mathbf{K} + \mathbf{L}) + (I_{175-165})_{na} + I_{210-190}$	2.79–2.82
5-5′ etherified ($\mathbf{T}$)$_{et}$	$1 - (I_{162-148})_{ac} + (I_{157-151})_{na} - \mathbf{E} - \mathbf{I}$	0.19
degree of cond.	(3.00 − h-units) − $[(I_{125-103})_{na} + \mathbf{M} + 2 \times \mathbf{I}]$	0.38
(conjugated CO/COOR)$_{et}$	$[(I_{162-148})_{ac} - 1.00 + \mathbf{E}] - (\mathbf{L} + \mathbf{S}$[a] $+ \mathbf{I})$	0.06
(conj. 5-5′)$_{ne}$ ($\mathbf{T}_a$)	$(I_{125-103})_{ac} - (I_{125-103})_{na} - \mathbf{K}$	0.08
minimum	$(\mathbf{L} + \mathbf{M} + \mathbf{R}) -$ conj.$_{et}$	0.05

[a] See Discussion. [b] As the structures are symmetric, their amount is half of the C_9 units involved. $(I_{i-j})_{na}$ and $(I_{i-j})_{ac}$ are integral values in the region of (*i*–*j*) ppm in the spectra of nonacetylated and acetylated lignins, correspondingly.

structures to be detected. Therefore, we attempted to quantify various lignin moieties on the structural level.

Carbonyl Structures. Integration of the 196−193 ppm (0.06/Ar) region embodies carbon atoms of carbonyl groups in coniferaldehyde (**L**) and α-CO/β-O-4 (**D**) moieties. The amount of the former has been estimated by the resonance at 9.75−9.55 ppm in the ^1H spectrum of the MWL-Ac using as a reference the resonance of aromatic protons or protons in acetyl groups determined by ^{13}C NMR (**Table 3**). Calculation using both integral references estimates the amount of coniferaldehyde as 0.04/Ar. Therefore, the amount of **D** moieties is ∼0.02/Ar. The amount of vanillin (**M**) moieties (resonance of C-α at 193−191 ppm) is about 0.05/Ar. This is somewhat higher as compared to the values reported earlier (**Table 1**) and can be attributed to a more intensive dry milling than that in toluene.

The signal at 182 ppm, 0.02/Ar (**Tables 2** and **3**), has been assigned to spirodienone structures (**I**) (*15*, *31*). Although quinone structures also resonate in this area, their amount in spruce MWL is very low (*2*, *15*).

Phenylcoumaran, Pinoresinol, and β-1 Structures. The amount of phenylcoumaran and β-1 structures was calculated by the method suggested earlier (*16*). The peak of the phenylcoumaran structure (**E**) is well-resolved at about 87 ppm in the spectrum of MWL-Ac, and integration gives a value of 0.09/Ar. As the integral at 50−48 ppm (0.10/Ar) embodies the phenylcoumaran and β-1 (**H**) structures, it follows that the amount of the latter is about 0.01/Ar. The number of C-β involved in pinoresinol structures (**F**) was approximately evaluated as 0.04/Ar by subtracting the amount of the phenylcoumaran structures from the integral at 54−52 ppm in the spectrum of the nonacetylated MWL. Because one pinoresinol structure involves two C-β atoms, the amount of these subunits is 0.02/Ar (**Table 4**).

β-O-4 Moieties. For discussion of the amount of β-O-4 moieties, it is important to specify the type of functional group at the α-position. In contrast to wet chemistry methods, NMR spectroscopy enables one to distinguish between β-O-4 structures with -OH, -CH_2-, -C═O, -O-Aryl, and -O-Alk groups at the α-position, dibenzodioxocin, and other moieties (*20*). The HMQC spectrum of the MWL shows only the presence of β-O-4 units of the types **A**, **C**, **D**, and **G**.

Dibenzodioxocin Structures (G). Dibenzodioxocin structures have been recently suggested to play an important role in lignin (*32*). Zhang and Gellerstedt estimated the amount of the dibenzodioxocin moieties as 0.05/Ar using quantitative HSQC (*17*). The signal at 83−81.5 ppm in the MWL-Ac spectrum (**Table 3**) corresponds exclusively to dibenzodioxocin structures. However, it is partly overlapped with a strong signal centered at ca. 79 ppm (**Figure 3**) and its integration gives an overestimated value. Therefore, a more accurate value for the amount of the dibenzodioxocin structures (0.07/Ar) was obtained subtracting the amount of pinoresinol moieties from the value of the integral at 86−83 ppm (**Table 4**). Argyropoulos et al. (*33*) reported a value of ∼0.04/Ar using DFRC/^{31}P NMR. This number was obtained by dividing the amount of 5-5 phenolic moieties liberated after DFRC treatment, 0.12/C_9 unit, by 3. However, only two phenolic OHs are liberated in cleavage of one dibenzodioxocin structure. Therefore, the corrected value should be 0.12:2 = 0.06/C_9 unit, which is rather close to our results.

β-O-4/α-OH Moieties. The amount of β-O-4/α-OH (**A**) moieties was estimated from the resonance at 77−72.5 ppm in

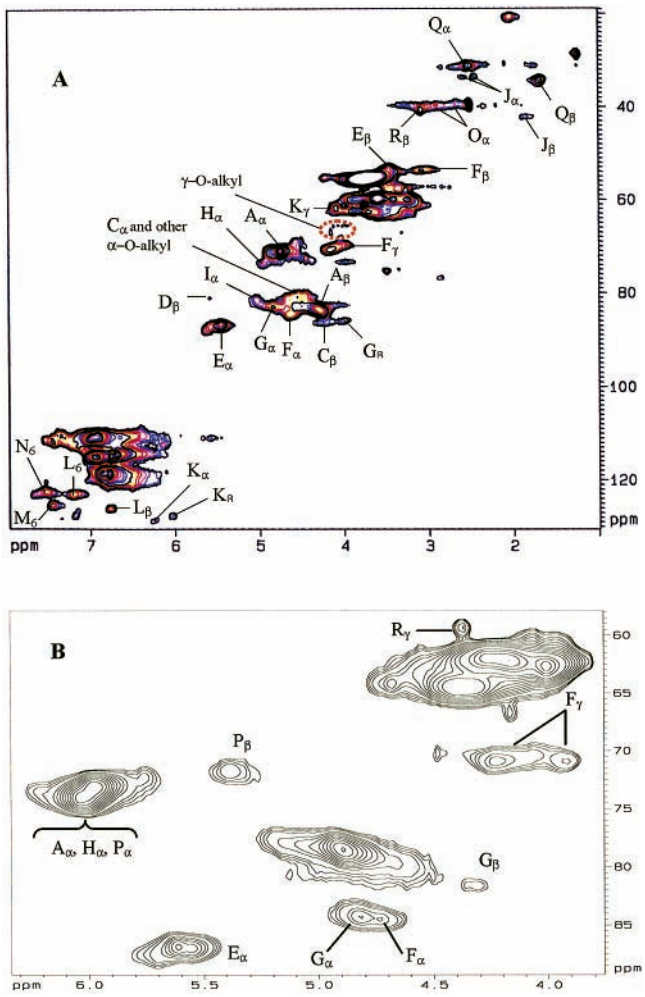

Figure 2. HMQC spectra of spruce MWL. (**A**) Nonacetylated and (**B**) acetylated, expanded, oxygenated aliphatic region.

the spectrum of MWL-Ac after correction for some minor moieties. According to the HMQC spectra (**Figure 2**), this region also includes β-1 (**H**) and arylglycerol structures (**P**). The latter were detected only in the HMQC spectrum of MWL-Ac indicating that their amount is close to the detection limit of the HMQC technique (about 0.01/Ar). The contribution of sugars to this area is about $3 \times 0.007 = 0.02$/Ar. Therefore, the amount of β-O-4/α-OH moieties is ~0.36/Ar. Moreover, there are two peaks in this area (**Figure 3**), corresponding to the *threo*- (74.5 ppm) and *erythro*- (73.1 ppm) isomers (*29*). The E/T ratio obtained, ca. 1:1, is consistent with literature data (*34*).

The total amount of identified β-O-4 moieties (**A**, **D**, and **G**) makes up 0.45/Ar. It should be noted that attribution of the integral at 62−58 ppm exclusively to β-O-4 moieties (excluding β-O-4/α-CO) from refs *10* and *35* results in their overestimation by 15−25% in the case of spruce MWL. However, this overestimation can be higher, for example, in a case of technical lignins (*35*) when a large amount of β-O-4 moieties is cleaved, and the contribution of other γ-OH groups to the 62−58 integral value is higher.

Coniferyl Alcohol Structures. It is rather difficult to precisely estimate the amount of coniferyl alcohol moieties (**K**) from ^{13}C spectra. Although the signal of C-γ of these structures can be clearly seen at ~61.8 ppm, it is overlapped by strong signals of C-γ in other γ-OH moieties, and the integration will give a significantly overestimated value. Therefore, the amount of coniferyl alcohol moieties, 0.02/Ar, was calculated by subtracting the amount of coniferaldehyde structures from the total amount of olefinic moieties in spruce MWL, 0.06/Ar (*2*, *5*).

Aliphatic Moieties. Dehydroconiferyl alcohol (**Q**) and sec-oisolariciresinol (**J**) structures were identified in the HMQC spectra (**Figure 2**) in accordance with recent publications (*36*); the contribution of other moieties in this region was very little. The amount of C-9 units involved in **Q** and **J** structures is about 0.02/Ar each (**Table 4**). From the resonance at 60−59 ppm in the spectrum of MWL-Ac, the amount of **R** moieties was estimated to be ~0.02/Ar (**Table 3**).

Functional Group Analysis. As shown above, our approach allows identification of the majority of lignin side chain structures on a structural level. In addition, these data can be substantiated by valuable information on the level of functional groups and various types of interunit linkages, as a whole.

Methoxyl Groups. The integral at 58−54 ppm is usually attributed exclusively to methoxyl groups (OMe). However, it also includes a small amount of other minor moieties (**Tables 2** and **3**). The amount of OMe groups after correction (**Table 4**) is 0.95/Ar, in good correlation with the value obtained by wet chemistry methods (*1*−*5*).

Carbonyl Groups. The amount of vanillin, coniferaldehyde, and α-CO/β-O-4 was estimated above. The peak centered at 197.5 ppm was originally attributed exclusively to C-α in structure **R** (*15*). However, other α-ketone moieties with exception of those of type **D** can contribute to this peak (*20*). The total amount of conjugated carbonyl moieties (integral at 200−190 ppm) is about 0.13−0.15/Ar. There is no noticeable peak in the region of nonconjugated CO groups (210−200 ppm) (**Figure 3**). However, small quantities of various moieties of this type might be present in the amount of ~0.03−0.05/Ar (**Tables 2** and **3**).

Carboxyl/Ester Groups. The amount of aliphatic and conjugated COOR groups was estimated from the spectrum of the nonacetylated MWL to be ~0.03/Ar and 0.02/Ar, correspondingly (**Table 2**). The HMQC spectrum (**Figure 2**) does not show any signals of CH-α in cinnamic acid derivatives at δ_C/δ_H 150−140/7.0−8.0 (*20*) indicating that the conjugated COOR groups are predominantly aromatic. The nature of the aliphatic COOR is not clear, although some of them are apparently of the Ar-CH_2-COOR type (**O**), corresponding to the signal of the CH-α observed in the HMQC spectrum.

Earlier works on the studies of spruce MWL with quantitative ^{1}H and ^{13}C NMR (*10*−*12*) reported very high amounts of carbonyl and ester groups. However, these values are not consistent with data obtained by wet chemistry methods (**Table 1**) or any other reports related to ^{13}C NMR of spruce lignin.

Hydroxyl Groups. The amount of hydroxyl groups was estimated from the 172−166 ppm region (*13*) of the ^{13}C NMR spectrum of the acetylated lignins (**Table 3**). The quantity of phenolic OH groups was corrected for the amount of Ar-COOR moieties resonating in the same area of the spectrum (**Table 4**). Although the total amount of aliphatic OH is consistent with an earlier publication (*13*), the amount of primary aliphatic OH in our lignin is slightly lower, and the amount of secondary OH is higher than the corresponding values of 0.30 and 0.78/OMe reported (*13*). One reason for the discrepancy may be due to the difference in lignin isolation after acetylation, as discussed above. This is consistent with the observation of decreasing amounts of α-OH groups in an acetylated MWL after purification of the acetylated preparation (*23*).

The amount of primary OH group can also be estimated from the cluster at 65−58 ppm. Its value (0.68−0.70/Ar) is very close to that for acetates of primary hydroxyl groups at 172−169.6

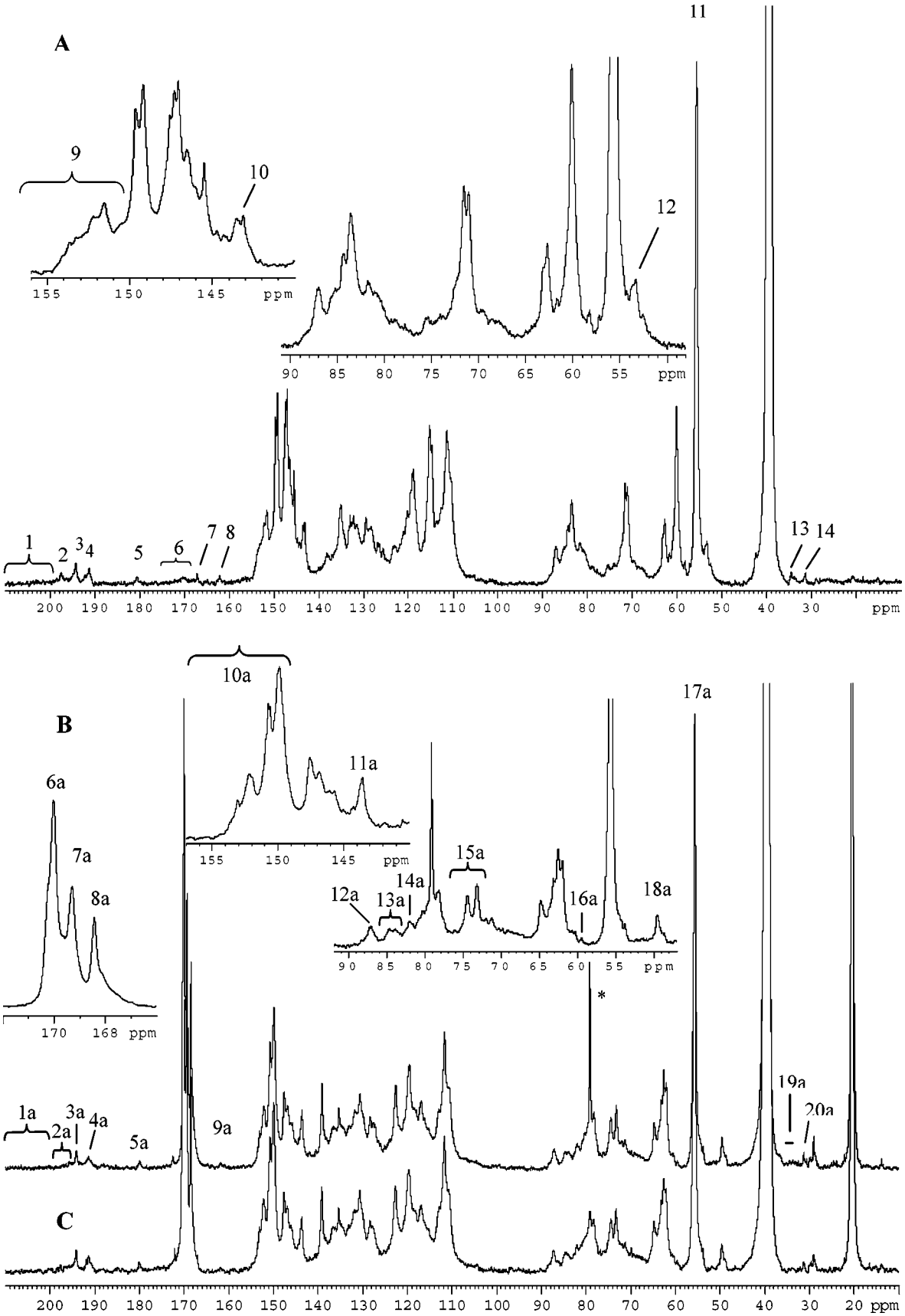

Figure 3. ^{13}C NMR spectra of spruce MWL. (**A**) Nonacetylated, (**B**) acetylated, and (**C**) acetylated MWL run at "classical" conditions (12 s pulse delay without relaxant). The lignin preparations on spectra **B** and **C** were acetylated separately; therefore, the spectra indicate not only good correlation between experiments with and without relaxant but also good reproducibility of the acetylation procedure. *Impurities of chloroform from sample handling were not integrated.

ppm in the spectrum of MWL-Ac. These values correlate with the sum of the γ-OH moieties identified (**A**, **D**, **E**, **G**−**K**, and **P**−**R**), 0.66/Ar. The amount of secondary OH groups is very close to the amount of benzyl alcohol groups estimated from

the region of 77−72.5 ppm in the spectrum of MWL-Ac, indicating that most of these groups in the MWL are α-OH.

γ-O-Alk Moieties. The cluster at 77−65 ppm embodies moieties with secondary OH groups and *γ-O*-Alk ethers. Subtracting the amount of secondary OH groups (**Table 4**) from the integral value of this region gives the amount of C-*γ* in *γ*-O-Alk ethers to be ∼0.24/Ar. From these moieties, only pinoresinol structures have been identified (∼0.04/Ar). The structure of other *γ*-O-Alk ethers (∼0.20 C-*γ*/Ar) is not known. The rather high content of *γ*-ether moieties estimated by ^{13}C NMR is consistent with NMR analysis of ginko MWL selectively enriched at the *γ*-position with ^{13}C (*37*), wherein ^{13}C NMR showed rather appreciable resonances at 70−65 ppm although no significant individual peaks were observed. Some weak signals at δ_C/δ_H 70−65/3.5−4.0, which can be attributed to *γ*-ether moieties, were detected in the HMQC spectrum (**Figure 2**). In fact, MWL analysis using an 800 MHz NMR spectrometer (*38*) showed other detectable signals in this area, not observable with lower field spectrometers. Thus, it is likely that a high variety of *γ*-ether moieties exists and results in a smearing of the resonance in this region.

Alkyl-O-Aryl and α-O-Alk Moieties. The cluster at 90−77 ppm consists of various alkyl-O-aryl moieties (α- and *β*-) and α-O-Alk moieties. As shown in **Table 4**, the amount of Alk-O-Ar ethers from the assigned moieties makes up 0.61/Ar. The total amount of C-4 etherified moieties is 0.67/Ar (**Table 4**). The difference between the etherified C-4 and the identified Alk-O-Ar structures, 0.06/Ar, can be attributed to unidentified Alk-O-Ar moieties and/or Ar-O-Ar moieties. Thus, the amount of Alk-O-Ar moieties is in the range of 0.61−0.67/Ar and the quantity of C-α atoms in α-O-Alk moieties is in the range of 0.15−0.21/Ar (**Table 4**). Among them, 0.08 C-α atoms/Ar are in pinoresinol and spirodienone moieties; the exact structure of the rest is not known. Some of the α-O-alkyl moieties have *β*-O-4 linkages (structures **C**), as evidenced by the signal of CH-*β* at δ_C/δ_H 86/4.3 in the HMQC spectrum of the nonacetylated MWL.

The inference of appreciable amounts of α-O-Alk moieties is consistent with ^{13}C NMR analysis of ginko MWL selectively enriched at the α-position with ^{13}C (*37*), which showed a rather intense resonance at ca. 82−78 ppm. The HMQC spectrum (**Figure 2)** does not show the presence of α-O-4 structures of type **B**, in agreement with published results (*25, 39*). In contrast, the signal of CH-α in benzyl-alkyl moieties has been detected at $\delta_C/\delta_H \sim$ 82/4.6 ppm. As the amount of carbohydrates in the MWL preparation is very low, the signal observed was assigned to benzyl-O-alkyl linkages between lignin units.

Balance of Side Chain Structures. The total amount of oxygenated carbon atoms (90−57 ppm) is about 2.12−2.17/Ar (**Tables 2** and **3**). This is lower than the value of 2.32/Ar reported earlier (*9*) but higher than expected from C-9 unit formula and functional group analysis (*1*).

The sum of the all carbon atoms in the side chains was estimated at 2.79−2.82/Ar (**Table 4**). The theoretical value for the phenylpropane unit is 3.00. The deficit of 0.18−0.21/Ar arises from moieties with short side chains, such as vanillin, Ar-COOR, and Ar-CH_2-COOR.

About 80% of the lignin moieties were assigned on the structural level (**Tables 1**−**4**). The exact structure of the remaining ∼0.20/Ar is not known. Most of the α- and *γ*-carbons in the unidentified structures are apparently involved in various alkyl-O-alkyl ethers. Some of the *β*-carbons can be of nonconjugated ketone (≤0.05/Ar), aliphatic carbon at 52−48 ppm (≤0.05/Ar), and a small amount of unidentified *β*-O-4 type moieties.

Aromatic Region. In the aromatic region, signals of different moieties are partially overlapped. In addition, some calculated values are obtained after subtraction of experimental or theoretical values, which are more than 1 order of magnitude higher than the value obtained; often, calculations require assumptions or/and the use of literature data (*9*−*12*). Therefore, we consider these calculations as approximate. However, this information, given the order of the magnitude, is useful, especially when it is confirmed by several independent methods.

The aromatic region of the lignin ^{13}C spectra is usually used for the estimation of different types of aromatic carbons: methine (C_{Ar-H}), quaternary oxygenated (C_{Ar-O}), and nonoxygenated (C_{Ar-C}). Among the quaternary aromatic carbons, it is important to quantify different condensed moieties such as 5-5 and 4-O-5 moieties of phenolic and etherified types. In addition, the content of *p*-hydroxyphenyl, guaiacyl, and syringyl units can be evaluated from the aromatic area of the ^{13}C spectrum.

p-Hydroxyphenyl Moieties. The amount of *p*-hydroxyphenyl moieties (h-units) in the MWL preparation (0.02/Ar) was estimated from the peak at 162−160 ppm in the spectrum of the nonacetylated lignin. No signal of syringyl units was detected in the HMQC or ^{13}C spectra indicating that their amount is negligible.

5-5′ Etherified Moieties. It is very difficult to distinguish between completely etherified, semietherified, and completely nonetherified (phenolic) 5-5′ condensed moieties because etherification/acetylation of the phenolic hydroxyl group affects the chemical shifts of only carbon atoms in this aromatic ring (ring I) (**Figure 1**, structure **T**) but not the adjusted one (ring II). Therefore, the terms "phenolic" and "etherified" 5-5′ moieties imply only one aromatic ring, not the whole 5-5′ structure.

The amount of 5-5′ etherified moieties was estimated earlier (*8*, *9*, *40*) by subtracting the amount of conjugated carbonyl structures from the integral at 157−151 ppm. In these calculations, it was assumed that all conjugated carbonyl structures are etherified. However, this approach with some necessary corrections gives a very low amount of 5-5′ etherified moieties, 0.09/Ar (Supporting Information), indicating that the assumption was incorrect. This also implies that the amount of phenolic conjugated carbonyl moieties is rather significant.

The amount of 5-5′ etherified moieties was approximately calculated from the integrals at 157−151 and 162−148 ppm in the spectra of the nonacetylated and acetylated MWL, correspondingly (**Tables 2** and **3**):

$$(I_{162-148})_{ac} - (I_{157-151})_{na} = (\text{all C-3} - \mathbf{E}_3 - \mathrm{h}_3 + \mathrm{h}_4 + \mathbf{S}_5 + \mathbf{L}_\alpha + \mathbf{I}_6 + \text{conj.}_{et4}) - (\mathbf{T}_{et3} + \mathbf{S}_{et3} + \mathbf{S}_{et5} + \mathbf{L}_\alpha + \mathbf{I}_3 + \mathbf{I}_6 + \text{conj.}_{et4}) = 1 - \mathbf{E}_3 + \mathbf{S}_{ne5} - \mathbf{T}_{et3} - \mathbf{S}_{et3} - \mathbf{I}_3$$

$$\mathbf{T}_{et} = 1 - (I_{162-148})_{ac} + (\mathrm{I}_{157-151})_{na} - \mathbf{E} - \mathbf{I} + \mathbf{S}_{ne} - \mathbf{S}_{et}$$

The amounts of 4-O-5 etherified and nonetherified moieties are rather small and about equal, ∼0.02/Ar of each (*2, 5, 9*). It follows that the amount of 5-5′ etherified units is

$$\mathbf{T}_{et} = 1 - (I_{162-148})_{ac} + (I_{157-151})_{na} - \mathbf{E} - \mathbf{I} = 1 - 1.07 + 0.37 - 0.09 - 0.02 = 0.19/\mathrm{Ar}$$

It is noteworthy that although the contribution of dibenzodioxocin moieties to this value is rather significant (∼0.14/Ar), there are still appreciable amounts of other 5-5′ etherified structures.

5-5′ Phenolic Moieties. The C-4 in nonconjugated 5-5′ phenolic moieties resonates at 144−142 ppm together with C-3 of phenylcoumaran structures (*9, 20, 40*). Acetylation of phenolic hydroxyl groups shifts the resonance of the C-4 atom

to a higher field. However, the integral value at 144−142 ppm is the same in the spectra of acetylated and nonacetylated MWL (**Tables 2** and **3**) indicating the absence of nonconjugated phenolic 5-5′structures in the MWL preparation. At the same time, other techniques, for example, permanganate oxidation (*1*−*5*) and ^{31}P NMR (*33*), did show a noticeable amount of these moieties in spruce lignins. If the 5-5′ phenolic structures are of conjugated CO/COOH type, the signals of C-4 shift to 150−146 ppm (*20*, *41*). Although exact estimation of these moieties is very difficult, a combination of a few independent approximate calculations gives the range of ∼0.05−0.08/Ar (**Table 4** and Supporting Information).

Oxygenated Aromatic Moieties. The resonance at 160−142 ppm (2.08/Ar) is usually assigned to oxygenated aromatic carbons (C_{Ar-O}). Because the theoretical amount of these atoms in G-units is 2.00/Ar, the difference 0.08/Ar is believed (*10*) to give the amount of Ar-O-Ar structures, such as 4-O-5, and syringyl units. However, C-4 in syringyl and 4-O-5 structures resonate at higher field (∼138 ppm) except those with conjugated CO/COOH (*20*). Moreover, C-α in coniferaldehyde moieties (∼153 ppm) also contributes to the C_{Ar-O} area. Correction for *p*-hydroxyphenyl units, which contribute with only one carbon atom to this region, is also required. Therefore, the calculation of Ar-O-Ar structures from this region of the spectrum (*10*) is incorrect.

Tertiary Aromatic Moieties. The region of 125−103 ppm (2.50/Ar) is usually attributed to aromatic methine carbons (C_{Ar-H}) (*7*−*12*). The theoretical value for C_{Ar-H} in noncondensed guaiacyl units is 3.00, and the difference between it and the integral at 125−103 ppm is usually considered as the degree of condensation. However, some corrections should be made. Only two carbons from *p*-hydroxyphenyl units resonate in this region, and the signal of C-6 in vanillin moieties is at 126 ppm (*20*). The chemical shifts of C-6 and C-5 in spirodienone moieties are also higher than 125 ppm (*31*), so the corrected degree of condensation is about 0.38/Ar (**Table 4**).

Correlation of the Information Obtained with Known Database. *Correlation with Wet Chemistry Methods.* The results obtained were compared with different models of spruce MWL structure acquired from reviewing different wet chemistry techniques (*1*−*3*) (**Table 1**). As seen, the approach suggested is very efficient in providing information comparable with the whole set of techniques used before and in some cases provides exclusive information (**Table 1**). A limitation of the NMR method is the problem in the quantification of 4-O-5 and β-6 moieties, which, however, are of low abundance in the spruce MWL (*2*).

Generally, the NMR approach gives results rather close to those obtained by wet chemistry techniques. However, there are some points of contradiction. One of the important issues is that the amount of benzyl alcohol groups estimated by NMR is more than twice that obtained before (**Table 1**), a discrepancy indicated earlier (*13*). The benzyl alcohol groups were detected by DDQ oxidation of permethylated lignin followed by UV detection of the α-carbonyl groups formed. Our preliminary studies on this reaction with the HMQC technique showed that the reaction is not selective; the oxidation of α-OH groups is not complete and some yet unidentified structures are formed in notable amounts in addition to the target α-carbonyl products. Therefore, we believe that NMR techniques give more reliable values than the previous method. It is important to note that the amount of benzyl alcohol OH cannot be lower than the amount of β-O-4/α-OH moieties (**A**). In some models, this obvious fact is not consistent.

Another issue is the origin of phenolic OH group liberated during mild acid hydrolysis of MWL. Originally, this phenomenon was attributed to the presence of noncyclic α-O-4/β-O-4 moieties (type **B**). Correlation NMR techniques showed the absence of structures of this type in lignin. Instead, it was suggested that α-O-4 structures are present in the form of dibenzodioxocin moieties (*32*). To correlate NMR conclusion with the results obtained from mild acid hydrolysis of MWL (*2*), we suggest that α-O-4 bonds in dibenzodioxocin structures are hydrolyzed under these conditions. The amount of dibenzodioxocin moieties estimated in our work is in good correlation with the number of 0.06−0.08 phenolic OH per C-9 unit liberated in the acid hydrolysis (*2*).

The nature of alkyl-O-alkyl moieties suggested in this paper is not completely explored. It is noteworthy that the presence of these moieties in MLW was considered by a few researchers (*1*, *3*, *25*, *42*) and doubted by others (*2*, *5*, *43*) as an experimental artifact. One of the reasons for this uncertainty is the difficulty in detection of these moieties by such degradation techniques as acidolysis due to hydrolysis of ether structures. Another reason is apparently high varieties of alkyl-O-alkyl structures. Therefore, even the models considering the presence of alkyl-O-alkyl ethers (*1*, *3*) give numbers lower than estimated from our NMR results. This matter needs further investigation. The discrepancies in the estimation of the amount of β-1 moieties by different analytical methods were comprehensively discussed recently (*16*).

Correlation with Other NMR Calculation Methods. The approach suggested appreciably increased the amount of information obtained by the NMR analysis. Particularly, the analysis of only nonacetylated spruce MWL requires the use of information obtained from different degradation techniques (*8*, *9*). In contrast, the joint use of nonacetylated and acetylated lignins along with modifications made in the calculations allows comprehensive quantification of various lignin structures without using data from other analytical methods. Comparing our results with another approach for quantification of spruce MWL moieties using ^{1}H and ^{13}C NMR (*10*−*12*) revealed some important discrepancies (**Table 1**), which were discussed above.

Our results are rather close to quantification of the side chain structures using a quantitative HSQC NMR technique (*15*−*17*). Some small differences show deviation between these methods and/or are caused by different origins of the lignins. This has a 2-fold implication: (i) the quantification by the HSQC technique (*15*−*17*) is reliable and (ii) the same information can be obtained with the more feasible ^{13}C NMR experiment. The quantitative 2D NMR could be an ideal experiment for the estimation of specific lignin structures in the side chain. However, only the ^{13}C NMR approach provides information on the quaternary carbons. In addition, ^{13}C NMR gives valuable information about the different types of functional groups and interunit linkages as a whole, contrary to 2D techniques.

Finally, we can conclude that the approach suggested here gives a comprehensive and rather reliable picture of the structure of lignin, compatible with the whole set of other methods in lignin chemistry. Moreover, it is important that all of this information can be obtained in relatively short experimental time and with a small amount of sample. This approach can be used not only for spruce lignin and similar lignins of guaiacyl type but also for other types of lignins such as hardwood and grass lignins of S/G and S/G/H types. However, certain modifications in the calculation procedure can result from differences in lignin structures, which should be verified by correlation NMR techniques before the quantification.

ABBREVIATIONS USED

MWL, milled wood lignin; MWL-Ac, milled wood lignin acetylated; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; DEPT, distortionless enhancement by polarization transfer; DHP, dehydrogenation polymer; DFRC, derivatization followed by reductive cleavage; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; E/T, *erythro*- and *threo*- ratio.

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Supporting Information Available: Calculation of 5-5′ etherified moieties according to ref *9*, the nature of signals at 145 to 140 ppm, etherified conjugated carbonyl/carboxyl moieties, and phenolic conjugated carbonyl/carboxyl moieties and 5-5′structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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